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(54) Bacteriophage lysins and their applications in destroying and testing for bacteria.

(57) Bacteriophages of food-contaminating or pathogenic bacteria or the lysins thereof are used to kill such bacteria. Examples include lysins from bacteriophages of *Listeria monocytogenes* and *Clostridium tyrobutyricum*.

Tests for bacterial contamination can be made specific for specific bacteria by using the appropriate bacteriophage or lysin thereof and determining whether cells are lysed thereby.

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produc destined for consumption by humans or animals (such as stor d potatoes). In agriculture, a particular applicati n is addition to silage where *Listeria* and *Clostridium tyrobutyricum* are known to present a problem that can be passed on up the food chain. In br wing, br wing yeast transform d with a lysin gene may be used.

In a medical or veterinary context, becaus the lysin is likely to be d graded r to produce an immune reaction, it is preferred to administer it topically in diseases of the skin such as ulcers, burns and acne. It may be applied as the clinician directs, as a lotion, cream or ointment.

An eighth aspect provides a method of testing for the presence of bacteria which are lysed by a bacteriophage or by the lysin thereof, comprising exposing a sample to the said bacteriophage or lysin and determining whether bacteria have been lysed as a result of such exposure.

Any technology that exploits the release of intracellular biochemicals (eg ATP or enzymes such as alkaline phosphatase or esterase) to detect micro-organisms can, in accordance with the invention, be made specific for the target range of such lysins. For example, an ATP or phosphatase release test for *Listeria* using the *Listeria* bacteriophage or lysin thereof, in which the release of ATP or phosphatase is detected (eg by linkage to a luciferase reaction and monitoring of photon release or by spectrophotometric methods as is described below) indicates the specific presence of *Listeria* in a sample. The invention further provides a kit comprising a lysin and means to detect bacterial lysis.

Preferably, the bacteriophage in all these contexts is or at least includes *Listeria monocytogenes* øLM4 or a bacteriophage of *Clostridium tyrobutyricum*, such as øP1. Several different lysins may be used in order to destroy or identify a specific range of bacteria.

The cloning and characterization of the gene for the lysin of the *Listeria* bacteriophage øLM4 has facilitated the production of the free lysin and the availability of its structural gene. These components have application in the protection of environment and food material from pathogenic strains of *Listeria*. The free lysin acts as a novel antimicrobial that kills such bacteria and the gene can be genetically engineered in a non-pathogenic micro-organism such that the latter produce the *Listeria* lysin thereby equipping it with a novel anti-*Listeria* capability. For example, a food-grade micro-organism may be transformed with a DNA construct comprising a coding sequence for the lysin.

Preferred embodiments of the invention will now be described by way of example with reference to the accompanying drawings, in which:-

Figure 1 shows patches of *E. coli* clones with *Hin*dIII fragments of øLM4 DNA in the *Hin*dIII site of vector pUC18. The plate is overlayed with a suspension of *Listeria monocytogenes* 6868 cells and lysin producing clones create clear zones around the patch (indicated by an arrow).

Figure 2 is a restriction and deletion map of lysin-expressing clone pFI322. The result of lysin activity tests is indicated to the right. The inferred location of the lysin gene is shown. Arrows indicate the orientation of the lysin gene with respect to the *lac* α promoter of the pUC vector used which is transcribed from left to right in this figure (ie pFI324 is opposed to the *lac* α promoter, other clones are transcribed in the same direction as the *lac* α promoter).

Plasmid pF1322 is pUC18 carrying a 3.6kb *Hin*dIII fragment of bacteriophage øLM4 DNA. Plasmid pF1326 is pF1322 with a 0.56kb *Hin*dIII - *Sal*I deletion. Plasmid pF1327 is pF1322 with a 1.32kb *Hin*dIII - *Eco*RI deletion. Plasmid pF1324 is pUC18 carrying a 1.9kb *Hin*dIII - *Nru*I fragment of pF1322 cloned between its *Hin*dIII and *Hin*cII sites. Plasmid pF1325 is pUC18 carrying a 1.6kb *Nru*I - *Hin*dIII fragment of pF1322 cloned between its *Hin*cII and *Hin*dIII sites. Plasmid pF1328 is pUC19 carrying a 1.9kb *Hin*dIII - *Nru*I fragment of pF1322 cloned between its *Hin*dIII and *Hin*cII sites. Plasmid pF1329 is pF1328 carrying a 1.6kb BaβI deletion from the polylinker *Bam*HI site. Plasmid pF1330 is pF1328 carrying a 1.6kb BaβI deletion from the polylinker *Bam*HI site.

Figure 3 illustrates the response of a suspension of *Listeria monocytogenes* 6868 cells to cell free extracts of *E. coli* strains harbouring plasmids pFI322(▲), pFI328(Δ), pFI329(○) and pUC19(●).

Figure 4 is a Coomassie blue stained SDS polyacrylamide gel of proteins produced by *E. coli* strain carrying the T7 expression vector pSP73 (tracks 2 and 3) or pFI331 which carries the lysin gene (tracks 4 and 5). Uninduced cells (tracks 2 and 4) are compared with induced cells (tracks 3 and 5). Molecular weight markers are present (tracks 1 and 6) and the expressed lysin protein is indicated by an arrow.

Figure 5 illustrates the sequencing strategy used. The extent and direction of sequences determined are indicated by the arrows. Synthetic oligonucleotide primers are indicated by boxes.

Figure 6 shows a single strand of the region of øLM4 DNA that encodes the lysin gene.

Figure 7 is the Analyseq print out of the analysis of the DNA sequence shown in Figure 6. The identification of the open reading frame of the lysin gene is in the top panel.

Figure 8 shows the double stranded DNA s quence of the lysin structural g ne and its translat d protein product.

Figure 9 shows the prot ctiv effect of cl n d *Listeria* lysin on skimmed milk to which *Listeria Monocytogen s* is added.

as the plate assay described above and illustrated in Figure 1, a sp ctrophotometric assay was also us d. For this the *E. coli* strain carrying plasmid clones wer grown at 37°C for 18 hours, harvested by centrifugation at 6000 x g for 5 min at 4°C, wash d down once in 100mM Tris buffer pH7.5 and resuspended in this same buffer at approximately 10mg dry weight/ml. Cell fre extracts were mad by 6 cycles of ultrasonication (15 sec on, 10 sec off) at 0°C using the microprobe of an MSE Soniprep 150. Unbroken cells and cell debris were removed by centrifugation at 25000 x g for 15 min at 4°C.

Samples of the cell free extracts were added to an equilibrated (5 min at 37°C) 4ml reaction mixture containing 400µmole Tris HCl pH7.5 and *Listeria monocytogenes* F6868 indicator cells that had been harvested and resuspended at an O.D. 600 of 2.3. The fall in optical density caused by lysis of indicator cells was followed using a spectrophotometer. Typical results from use of this protocol are presented in Figure 3. The lytic activity of the plasmid derivative described above and in Figure 2 were assessed using both of these methods and the results are presented in Figure 2.

These results demonstrated that the structural gene for bacteriophage øLM4 was contained within the left hand 1.2kb of the DNA cloned in pFI322 and defined by the *Hin*dIII site at co-ordinate 0 and the *Eco*R1 site at co-ordinate 1.25 of the map illustrated in Figure 2.

Figure 2 also indicates the orientation of *Listeria* bacteriophage øLM4 DNA with respect to the *E. coli lac* α promoter that is present on vectors pUC18 and pUC19. It is apparent that a positive reaction in the lysin assay is only found when one orientation is maintained (eg pFI324 is negative whereas pFI328 is positive even though both constructs contain the same *Listeria* bacteriophage øLM4 fragment). This suggests that expression of the lysin gene depends on use of the *E. coli lac* α promoter and that no *Listeria* bacteriophage øLM4 promoter is present and active in *E. coli*.

Detection of the lysin protein

In order to identify a protein produced by the fragment of øLM4 DNA that expressed lysin activity another *E. coli* vector was used. A 2kb fragment from plasmid pFI328 between the *Hin*dIII site at co-ordinate 0 and a unique *Bam*HI site present on the polylinker of pUC19 was isolated and cloned between the *Hin*dIII and *Bam*HI sites of the T7 expression vector pSP73 that was purchased from Promega. The constructed plasmid named pFI331 was transformed into the *E. coli* host strain JM109DE3.

The *E. coli* T7 promoter in this vector is expressed by the phage specific T7 RNA polymerase which is induced by addition of IPTG in the appropriate host strain *E. coli* JM109 DE3. Cultures of this strain carrying pSP73 as a control or pFI331 were grown for 3 hours and induced by addition of 1PTG to a final concentration of 0.2mM. Incubation was continued for a further 3 hours before the cultures were harvested and used to prepare cell extracts using well-established, published procedures (Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Methods in Enzymology* **185**: 60-89).

Proteins present in cell extracts were analysed using conventional SDS-polyacrylamide gel electrophoresis (Laemmli (1970) *Nature* **227**: 680-685). The results presented in Figure 4 clearly demonstrate that the 2kb fragment of pFI331 expresses a single protein with a molecular size of 31 kilodaltons which represents the lysin enzyme.

DNA sequence of the *Listeria* bacteriophage øLM4 lysin gene

The region of DNA between co-ordinate 0 and 1.2 in Figure 2 was subject to oligonucleotide sequence analysis using the dideoxy chain-termination method (Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) *J. Molec. Biol.* **143**) with a sequenase version 2.0 kit (United States Biochemical Corporation). The 0.9kb *Hin*dIII - *Eco*RI and the 0.3kb *Eco*RI - *Eco*RI fragments of pF1328 were subcloned in the M13 sequencing vectors M13mp18 and M13mp19 to create templates and sequenced using universal and synthetic oligonucleotide primers. To sequence across the internal *Eco*RI site at co-ordinate 0.9 double stranded sequencing of pFI329 plasmid DNA was used. The sequencing strategy is presented in Figure 5 and the complete DNA sequence is in Figure 6. The sequence was analysed using the computer programme ANALYSEQ (Staden (1980) *Nucleic Acid Research* **8**: 3673-3694) which revealed an open reading frame that represents the *Listeria* bacteriophage lysin gene. The printout from the Analyseq analysis is presented in Figure 7 and the open reading frame representing the lysin structural gene and its translated protein product is presented in Figure 8. The molecular size of the translated protein was calculated to be 32.9 kilodaltons which agrees well with the calculated 31 kilodalton size of the protein expressed by the T7 vector pSP73 (Clone pFI331 in Figure 4).

TABLE 1: ACTIVITY OF CLONED LYSIN AGAINST LISTERIA SPECIES

Organism	Strain	Serotype	Relative[a] Activity	Time (min)[b] $\Delta OD_{600}=1$
Listeria monocytogenes	F6868	4b	1.00	20
	NCTC 7973	1a	0.19	53
	NCTC 5412	4b	0.90	13
	F4642	4b	0.92	14
	NCTC10357	1a	0.92	20
	BL87/41	4	0.66	25
	NCTC 5348	2	0.10	78
	SLCC2373	3a	1.20	17
	SLCC2540	3b	0.19	60
	SLCC2479	3c	0.15	60
	SLCC2374	4a	0.54	30
	SLCC2376	4c	0.19	90
	SLCC2377	4d	0.08	90
	SLCC2378	4e	0.56	28
	SLCC2482	7	0.45	36
	L3056	1/2a	0.49	30
	L4203	1/2a	0.36	41
Organism	Strain	Serotype	Relative[a] Activity	Time (min)[b] $\Delta OD_{600}=1$
	L4490	1/2b	0.29	55
	L1378	1/2b	0.09	150
	L4281	1/2c	0.11	120
	L3304	1/2c	0.12	90
	L3253	4bx	0.66	26
	L2248	4bx	0.08	72
Listeria innocua	NCTC11288	6a	0.90	12
	NCTC11289	6a	0.69	22
Listeria ivanovii	NCTC11007		0.95	18

known N terminal secretory leader such as those of the proteinase gene, the *usp*45 gene or the nisin precursor gene of *Lactococcus lactis*. Suitable organisms for this application concept include strains of *Lactococcus lactis* in cheese and dairy products and *Lactobacillus plantarum* or *Pediococcus* species in agricultural silage.

The *Listeria* lysin gene from plasmid pF1328 was isolated together with its own ribosome binding site using the polymerase chain reaction. This fragment was cloned into the PstI site of *E. coli* vector pUC19 in both orientations (plasmids pF1531 and pF1532). Expression of this gene in *E. coli* strains was observed from one orientation only, under the control of the *lac* α promoter of the vector (plasmid pF1531). Enzyme activity of cell extracts of this strain was comparable to that of *E. coli* strains carrying plasmid pF1322. Using plasmid pF1532 that did not express the lysin gene and cloning the lactococcal *lac*A promoter/lacR gene on a *Bam*HI fragment (Van Rooijen *et al*, (1992) *J. Bacteriol.* **174**: 2273-2280) upstream of the lysin gene (plasmid pF1533) expression in *E. coli* of øLM-4 lysin from the lactococcal lacA promoter was obtained. The lytic activity of extracts from these *E. coli* strains was lower when the lysin gene was expressed from the lacA promoter. The *Sst*I/*Sph*I fragment of pF1533 containing the øLM-4 lysin gene with the lacA promoter/lacR gene was cloned into the *Sst*I/*Sph*I sites of the lactococcal vector pTG262 (Shearman *et al* (1989) *Molecular and General Genetics* **218**: 214-221) and the resulting plasmid pFI534 was used to transform *L. lactis* MG5267. As shown in Figure 10 cell extracts of this strain expressed øLM-4 lysin activity when grown on lactose, on glucose enzyme activity of cell extracts was reduced.

The øLM-4 lysin gene together with the lacA promoter/lacR gene was cloned into pF145, a plasmid expressing the *Lactococcus* phage øvML3 lysin gene which causes lysis during stationary phase of *L. lactis* cultures carrying the plasmid (Shearman et al (1992) *Biotechnology* **10**: 196-199). The resulting plasmid pF1535 in *L. lactis* MG5267 when grown on lactose produced a culture that grew to stationary phase, then lysed as a consequence of the øvML3 lysin, releasing øLM-4 lysin into the culture supernatant.

EXAMPLE 3: SPECIFIC DETECTION OF MICRO-ORGANISMS

The specificity of a bacteriophage lysin provides an opportunity to specifically detect those micro-organisms which are susceptible to it. For example to detect *Listeria sp.* the lysin described here may conveniently be used at a post enrichment stage where a broth culture of those micro-organisms present in a test sample is first produced. The identity of species of bacteria in the sample at this stage is unknown. The bacterial culture may be centrifuged and resuspended in an assay buffer (eg the one used here in studies of lysin specificity). A control preparation and separately a preparation containing active *Listeria* lysin are then added. Sufficient units of lysin activity are used to provide very effective lysis of any lysin susceptible cells (ie *Listeria*). After incubation for a short period (eg 30 min) any *Listeria* present will lyse, but other species will not. The presence of *Listeria* will then be detected by the lysis of bacteria in the sample treated with the lysin whereas no lysis occurs in the control.

The detection of lysis may be achieved by assaying an intracellular enzyme or metabolite. Especially useful enzyme assays are for phosphatase or for esterase. Alkaline phosphate can be assayed spectrophotometrically by following appearance of p-nitrophenol, which is yellow, from the colourless substrate p-nitrophenyl-phosphate at 405nm. Esterase activity can be assayed using fluorescein diacetate which is cleaved to acetate and fluorescent fluorescein and measuring the latter in a fluorometer. One especially suitable metabolite assay involves ATP detection. For this the well established luciferase assay in which ATP molecules generate light is exploited. Light emission may be measured in a luminometer. (An example of an end point detection reagent using luciferase-luciferin is marketed by Sigma Chemical Company as product L-1761).

EXAMPLE 4: *CLOSTRIDUM TYROBUTYRICUM* BACTERIOPHAGE øP1 LYSIN

Bacteriophage øP1 was isolated from a landfill core sample using *Clostridium tyrobutyricum* NCFB 1755 as host. Bacteriophage øP1 was tested against six more strains of *C. tyrobutyricum*. Strains NCFB 1753 and NCFB 1756 supported the growth of bacteriophage and they were thus host strains as was the strain NCFB 1755. Against *C. tyrobutyricum* strains NCFB 1715, NCFB 1754, NCFB 1757 and NCFB 1790 an undiluted øP1 stock suspension gave a clear zone but diluting out did not result in individual bacteriophage plaques. This indicates that these strains were lysin sensitive but not bacteriophage sensitive. Bacteriophage øP1 thus produces a lysin with a broad specificity for strains of *C. tyrobutyricum*. Similar tests of bacteriophage øP1 with a wide variety of other bacteria showed no effect of the lysin or bacteriophage particles against *C. sporogenes* strains ATCC 17886, NCFB 1789, NCFB 1791; *C. butyricum* strains NCFB 1713, NCFB 857; *Lactobacillus buitineri* strains NCFB 110, F3327; *L. brevis* strains NCFB 1749, F3328; *L. helv ticus* strains NCFB 1243, CNRZ 832; *L. bulgaricus* CNRZ448; *L. plantarum* strains NCFB 1752, NCFB 82, NCFB 963; *Escherichia coli* BL 90/12; *Bacillus cereus* NCTC 1143.

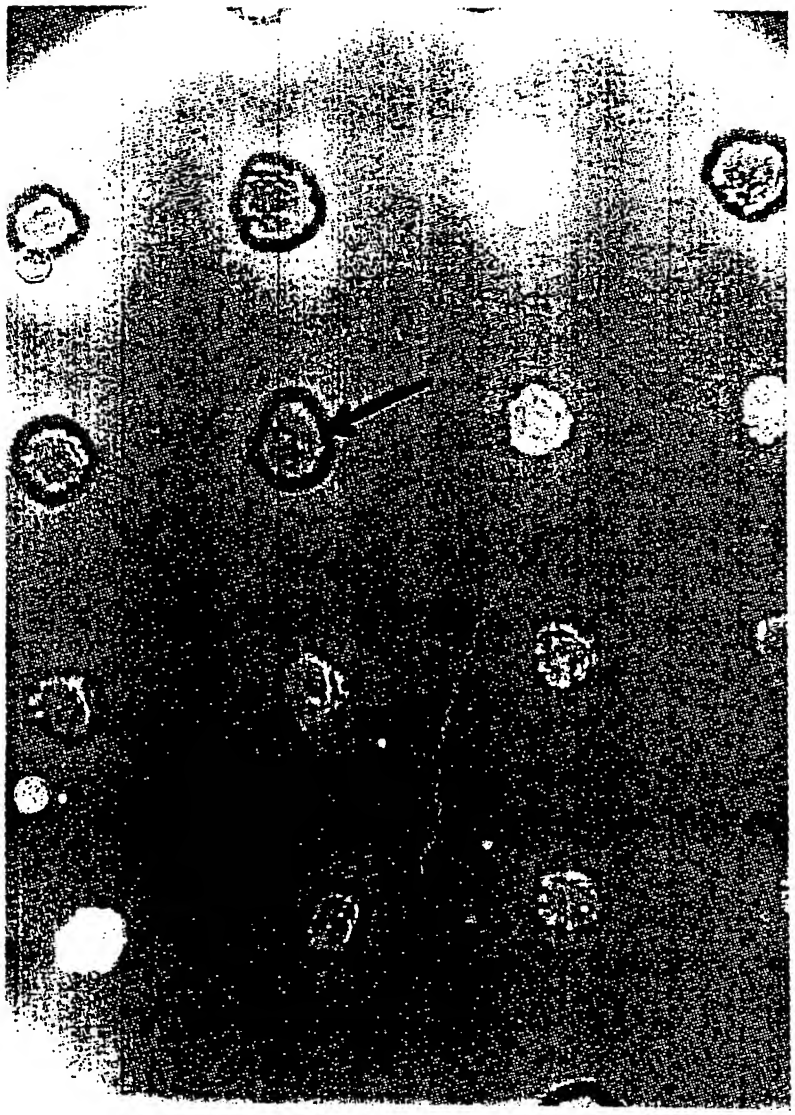

Fig. 1

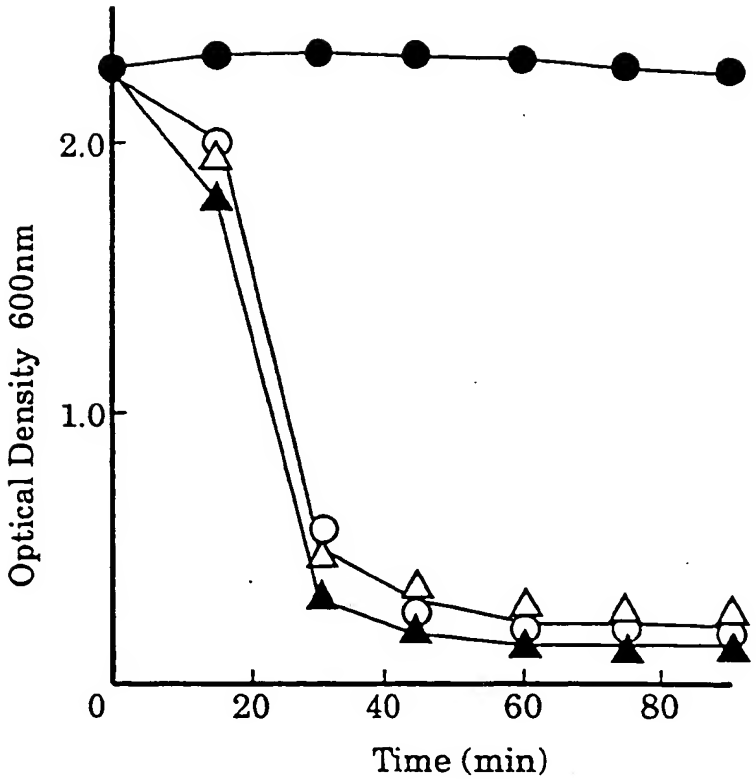

FIGURE 3

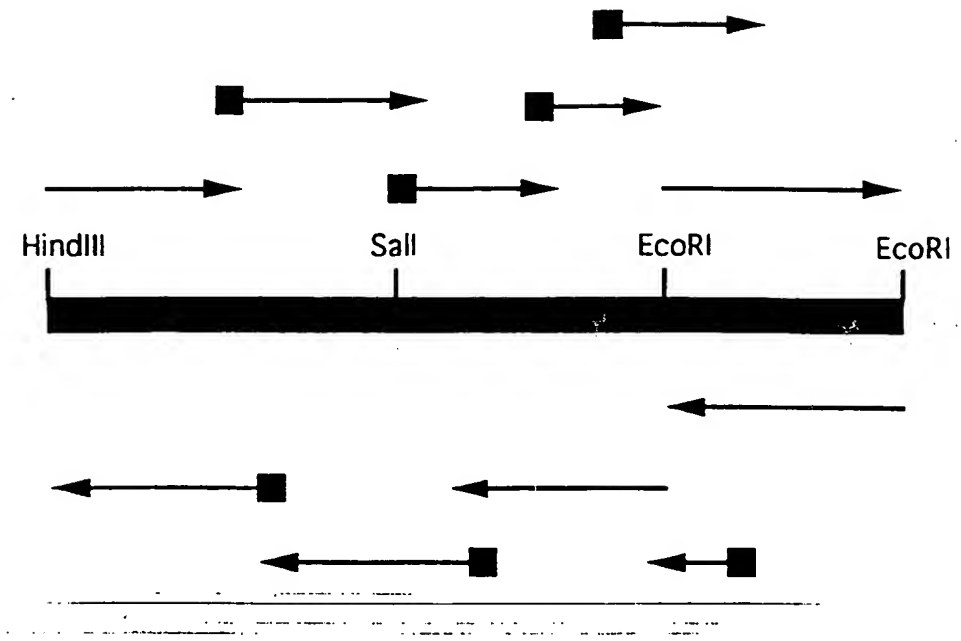

FIGURE 5

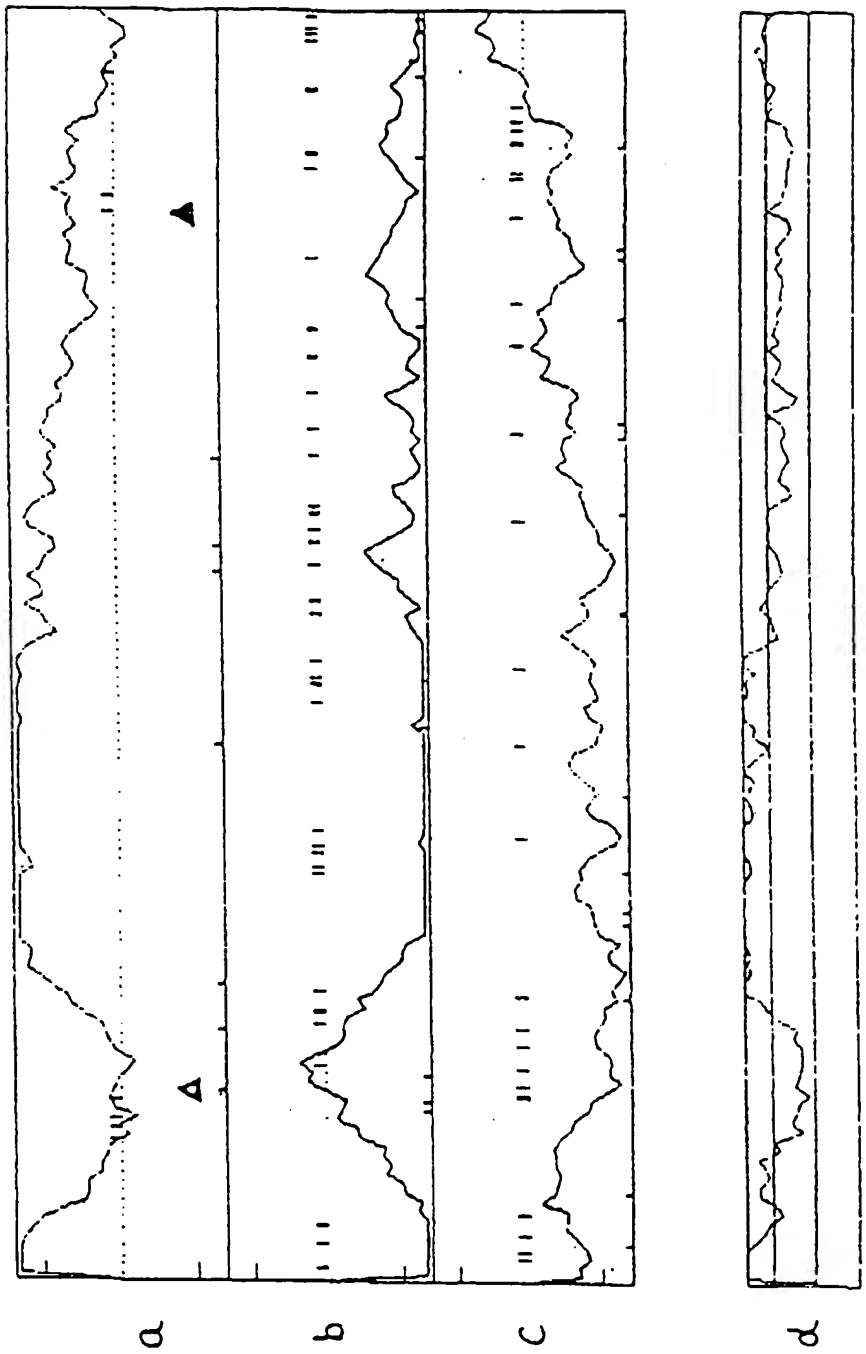

FIGURE 7

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      IleAsnAspLysLeuTyrTyrMetTyrLysSerPheCysAspValValAlaLysLysAsp
      ATCAACGACAAATTATACTACATGTATAAGAGCTTTTGCGATGTTGTAGCTAAAAAAGAT
786   ----+---------+---------+---------+---------+---------+-----  845
      TAGTTGCTGTTTAATATGATGTACATATTCTCGAAAACGCTACAACATCGATTTTTTCTA

      AlaLysGlyArgIleLysValArgIleLysSerAlaLysAspLeuArgIleProValTrp
      GCAAAAGGACGCATCAAAGTTCGAATTAAAAGCGCGAAAGACTTACGAATTCCAGTTTGG
846   ----+---------+---------+---------+---------+---------+-----  905
      CGTTTTCCTGCGTAGTTTCAAGCTTAATTTTCGCGCTTTCTGAATGCTTAAGGTCAAACC

      AsnAsnThrLysLeuAsnSerGlyLysIleLysTrpTyrAlaProAsnThrLysLeuAla
      AATAACACAAAATTGAATTCTGGGAAAATTAAATGGTATGCACCCAATACAAAATTAGCA
906   ----+---------+---------+---------+---------+---------+-----  965
      TTATTGTGTTTTAACTTAAGACCCTTTTAATTTACCATACGTGGGTTATGTTTTAATCGT

      TrpTyrAsnAsnGlyLysGlyTyrLeuGluLeuTrpTyrGluLysAspGlyTrpTyrTyr
      TGGTACAACAACGGAAAAGGATACTTGGAACTCTGGTATGAAAAGGATGGCTGGTACTAC
966   ----+---------+---------+---------+---------+---------+-----  1025
      ACCATGTTGTTGCCTTTTCCTATGAACCTTGAGACCATACTTTTCCTACCGACCATGATG

      ThrAlaAsnTyrPheLeuLys
      ACAGCGAACTACTTCTTAAAA
1026  ----+---------+------  1046
      TGTCGCTTGATGAAGAATTTT
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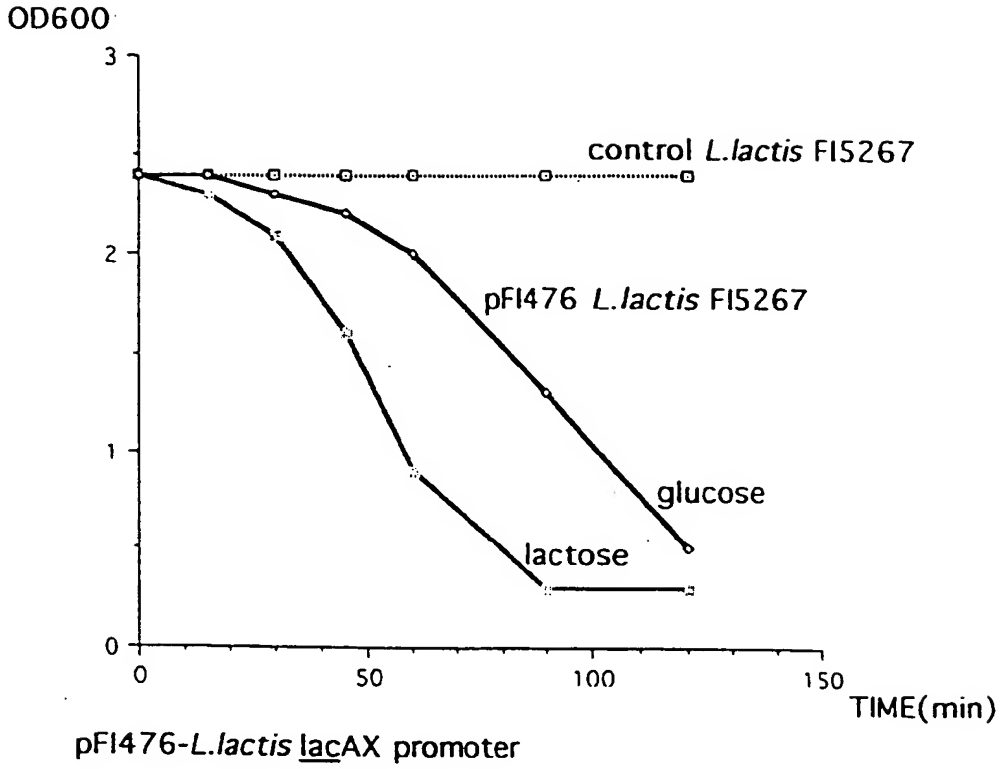
FIGURE 8 (END)



pFI476-*L.lactis* lacAX promoter

FIGURE 10

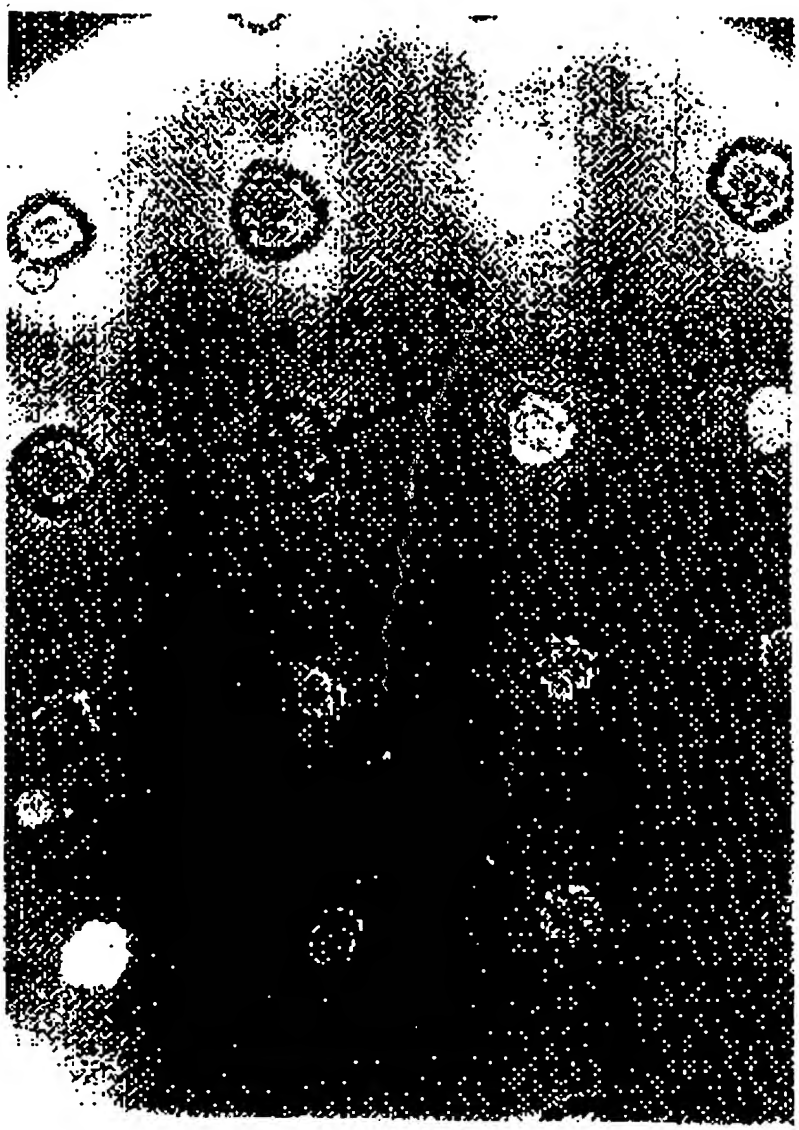


Fig. 1

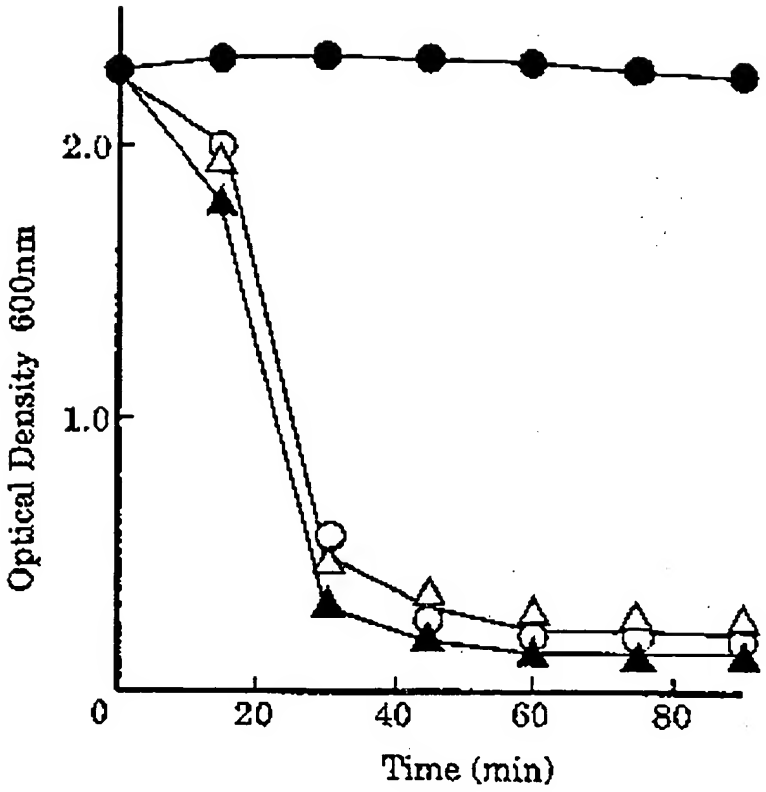


FIGURE 3

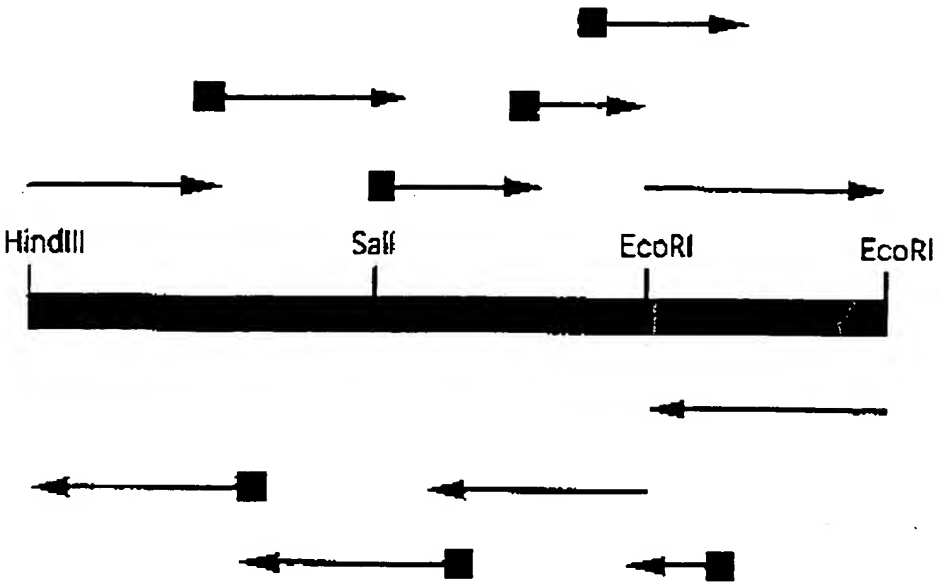


FIGURE 5

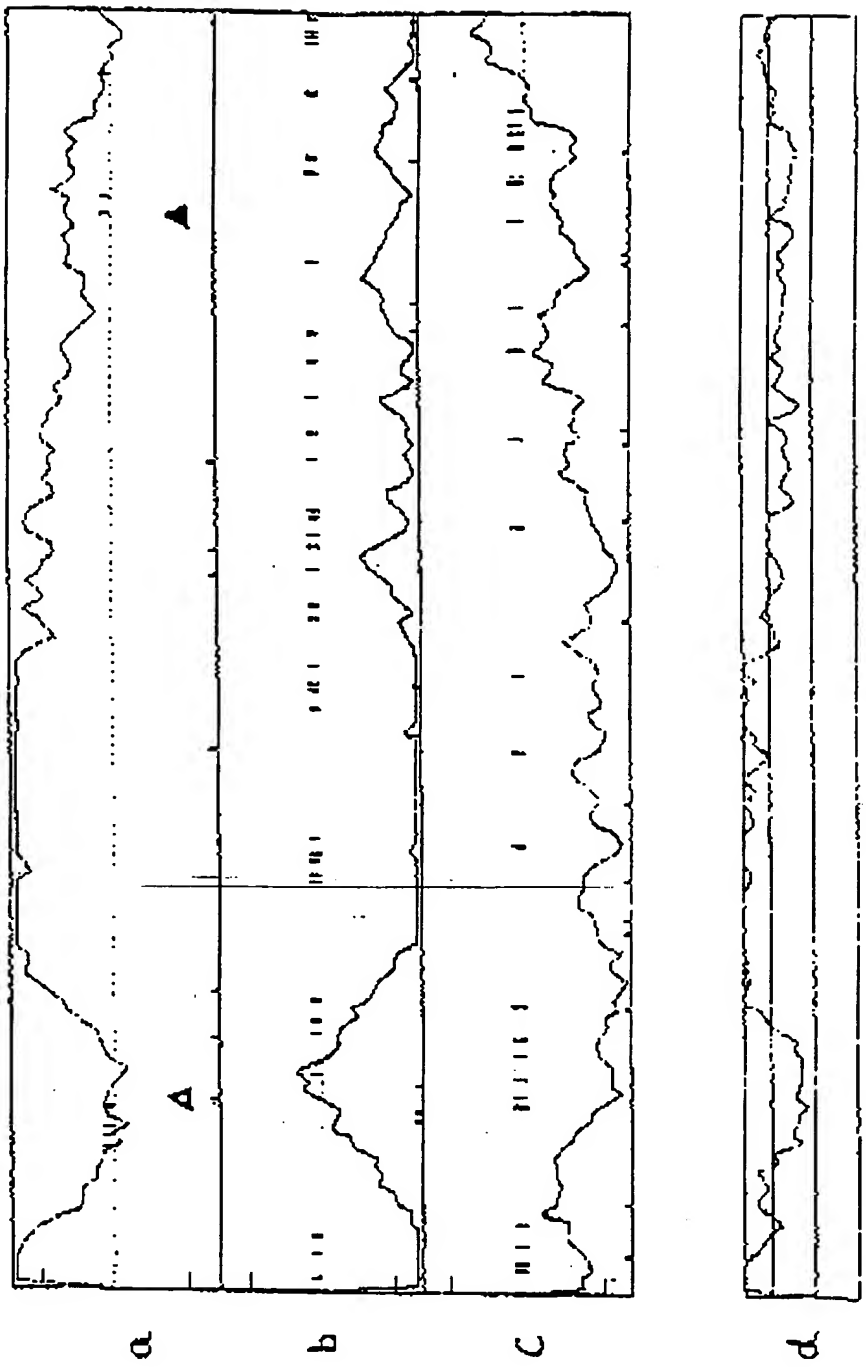


FIGURE 7

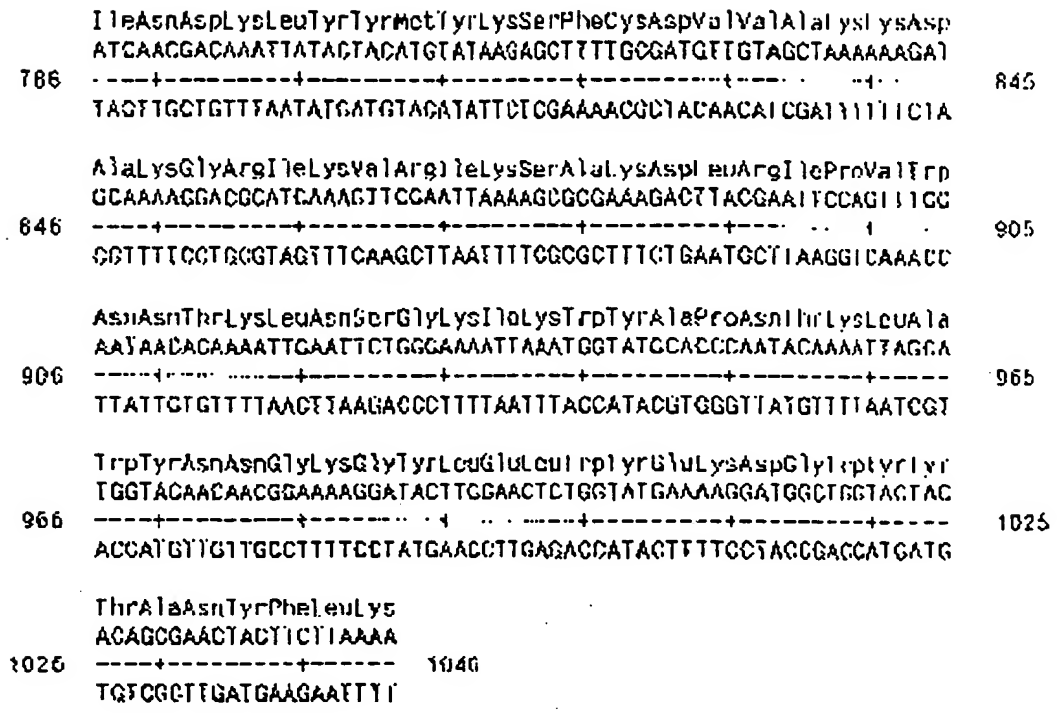
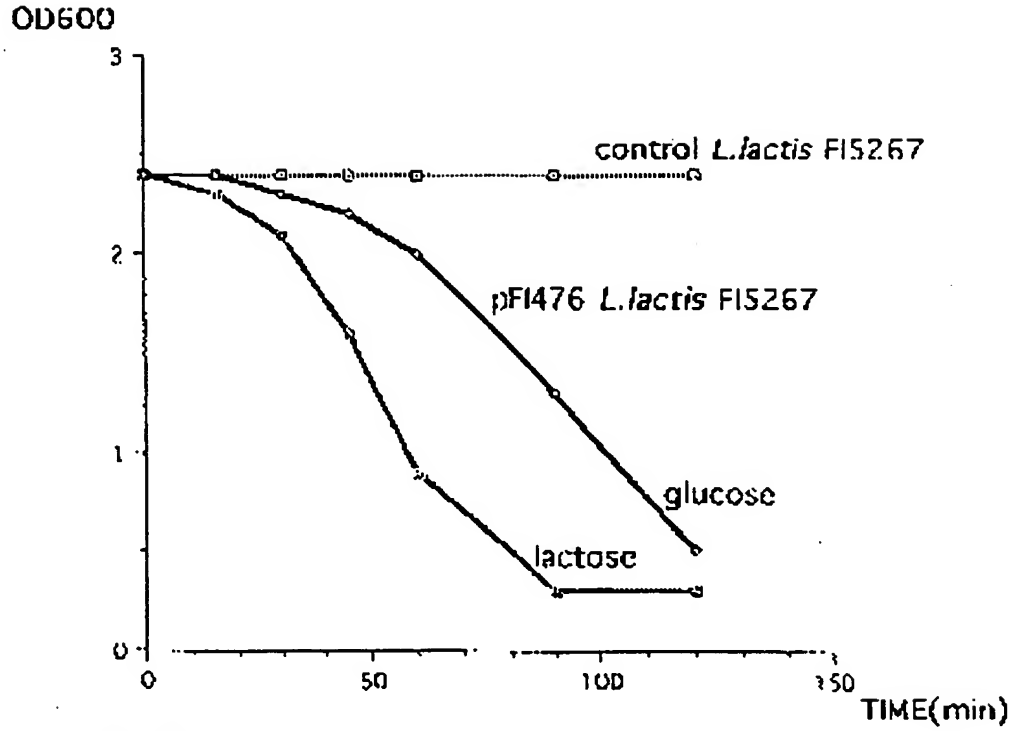


FIGURE 8 (END)



pFI476-*L.lactis* lacAX promoter

FIGURE 10